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Vaccinia Virus Serpins B13R (SPI-2) and B22R (SPI-1) Encode M_r 38.5 and 40K, Intracellular Polypeptides That Do Not Affect Virus Virulence in a Murine Intranasal Model

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A characterization of genes B13R (SPI-2) and B22R (SPI-1) from vaccinia virus strain Western Reserve (WR) is presented. These genes are transcribed early during infection and the predicted encoded proteins show similarity to the superfamily of serine protease inhibitors (serpins). The 5′ transcriptional initiation site of each gene was mapped by primer extension experiments to 71–72 and 31 nucleotides upstream of the B13R and B22R open reading frames (ORFs), respectively. Each ORF was expressed in *Escherichia coli* and specific antisera were raised against the protein produced. These antisera were used to identify the B13R- and B22R-encoded proteins in vaccinia virus-infected cells as stable, intracellular, nonglycosylated proteins of M_r 38.5K and M_r 40K, respectively. The B22R gene product was detected in all orthopoxviruses tested including cowpox, rabbitpox, and vaccinia strains WR, Copenhagen, Tashkent, Tian Tan, Lister, Wyeth, IHD-J, and IHD-W. In contrast, the B13R gene product had a more limited distribution and was not detected in Copenhagen, Tashkent, Lister, and Tian Tan. Viable virus deletion mutants that lacked only B13R or B22R coding sequences (ΔB13R and ΔB22R) and revertant viruses in which the deleted gene was restored were constructed by transient dominant selection. The growth of the deletion mutants in cell culture was indistinguishable from that of wild-type virus. Additionally the virulence of each deletion mutant was indistinguishable from wild-type and revertant viruses in a murine intranasal model.

INTRODUCTION

Poxviruses are large, complex viruses that replicate in the cytoplasm of infected cells and encode their own enzymes for transcription and DNA replication (Moss, 1992). The 191-kb dsDNA genome of vaccinia virus strain Copenhagen has been sequenced and encodes approximately 200 genes (Goebel *et al.*, 1990) of which approximately one-third are nonessential for virus replication (Johnson *et al.*, 1993). Several of these nonessential genes contribute to virus virulence *in vivo* and interfere with the host response to infection (Smith, 1993). Serine protease inhibitors, or serpins, are members of a superfamily of structurally related proteins. In mammals, many serpins are secreted into the plasma where they function as inhibitors of the serine proteases involved in the processes of inflammation, complement activation, and blood coagulation (Carrell *et al.*, 1987). In view of this, the presence within a number of poxvirus genomes of open reading frames (ORFs) with sequence homology to serpins (Pickup *et al.*, 1986; Boursnell *et al.*, 1988; Tomley *et al.*, 1988; Kotwal and Moss, 1989; Smith *et al.*, 1989b; Upton *et al.*, 1990; Massung *et al.*, 1993; Shchelkunov *et al.*, 1993) is of interest, as it seems likely that the gene products may influence virus pathogenesis.

The most extensively studied orthopoxvirus serpin is the cowpox 38K protein (also known as SPI-2 or crmA) which has been shown to have several functions. It is responsible, at least in part, for the hemorrhagic pock phenotype produced on the chorioallantoic membranes (CAMs) of fertile hens' eggs by cowpox virus (Pickup *et al.*, 1986; Palumbo *et al.*, 1994) and directly or indirectly prevents the migration of white cells *in vivo* (Palumbo *et al.*, 1989) and *in vitro* (Chua *et al.*, 1990). Additionally, it inhibits the *in vitro* processing of pro-interleukin-1β (pro-IL-1β) to mature, active IL-1β by the pro-IL-1β converting enzyme (ICE) (Ray *et al.*, 1992). This may confer the dual benefit of reducing release of proinflammatory IL-1β and inhibiting apoptosis which activated ICE induces in fibroblasts and neuronal cells (Miura *et al.*, 1993; Gagliardini *et al.*, 1994). The cowpox 38K protein also restricts the formation of a specific leukotriene from the lipoxygenase pathway of arachidonic acid metabolism (Palumbo *et al.*, 1993, 1994). Both IL-1β production and arachidonic acid metabolism are important components of the inflammatory cascade resulting from virus infection. Recently it has been shown that deletion of the cowpox 38K protein causes attenuation in a murine intranasal model (Thompson *et al.*, 1993).

A further example of the involvement of a poxvirus serpin in virus pathogenesis is given by the SERP1 gene

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of malignant rabbit fibroma virus and myxoma virus (both leporipoxviruses) which cause the rapidly fatal, immunosuppressive, systemic infection usually observed in infected rabbits. Deletion of this gene attenuates both viruses (Upton *et al.,* 1990; Macen *et al.,* 1993).

Three ORFs with sequence homology to serpins have been identified in the Western Reserve (WR) strain of vaccinia virus — the prototype orthopoxvirus. These are B13R and B22R (previously designated B24R (Smith *et al.,* 1989b)), located near the right end within the *Hin*dIII B fragment, and K2L situated near the left end within the *Hin*dIII K fragment. The B13R, B22R, and K2L gene products share 92, 46, and 19% amino acid identity with the cowpox 38K protein, respectively. The three WR ORFs are transcribed early during infection (Smith *et al.,* 1989b), but the encoded proteins have not previously been identified. However, it is assumed that K2L is translated because a fusion–inhibition phenotype has been associated with this gene (Law and Smith, 1992; Turner and Moyer, 1992; Zhou *et al.,* 1992).

In this report, a characterization of the B13R and B22R genes of vaccinia virus strain WR is provided. The 5′ ends of the early transcripts were mapped by primer extension, antibodies raised against the ORFs expressed in *Escherichia coli* were used to identify the proteins in vaccinia virus-infected cells, the distribution of these proteins in other orthopoxviruses was investigated, and the replication *in vitro* and virulen[illegible] mutants lacking these genes is d[illegible]

Characterization of infectious RYMV cDNA clones

MATERIALS AND METHODS

Cells and viruses

The WR strain of vaccinia virus was used throughout. This virus and derivative recombinants were grown on BSC-1 cells that were passaged in Dulbecco's modification of minimal essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Viruses were harvested from infected cells, sedimented through a sucrose cushion, and titrated by plaque assay on BSC-1 cells as described (Mackett *et al.,* 1985).

Plasmid constructions

Plasmids to be used for isolation by transient dominant selection of virus deletion mutants lacking either the B13R or B22R ORFs were constructed as follows.

B13R. Plasmid pSTH1, containing the right half of the vaccinia virus *Sal*I G fragment including most of the B13R ORF (Howard, 1991), was digested with *Hin*dIII, treated with Klenow enzyme, digested with *Sal*I, and then ligated with a 1188-bp fragment derived from the left end of the *Sal*I I fragment by *Bgl*II digestion, Klenow treatment, and *Sal*I digestion, forming plasmid pGS124. pGS124 was digested with *Eco*RI and *Bgl*II, treated with Klenow, and religated to form pYC15 which contained the intact B13R ORF and flanking sequences extending from the middle of B12R into B15R. pYC15 was digested with *Bam*HI, end-filled with Klenow enzyme, digested with *Hpa*I, and then religated. The resultant plasmid, pSK4, had lost an internal 926 bp (89%) of the B13R ORF (leaving 110 and 2 nucleotides at the 5′ and 3′ ends, respectively). A 1022-bp fragment spanning the deleted B13R gene was excised from pSK4 by digestion with *Xba*I and *Spe*I and ligated into *Xba*I-digested pSJH7 (Hughes *et al.,* 1991) that contains the E. coli guanine phosphoribosyl transferase (Ecogpt) gene under the P7.5 early/late promoter, generating pSK5. To make a revertant virus in which the mutated B13R gene of the B13R deletion mutant (ΔB13R, see below) was restored to wild type (WT), the intact B13R gene was cloned into pSJH7 by digestion of pYC15 with *Xba*I, partial digestion with *Spe*I, and ligation of a resultant 1948-bp fragment into *Xba*I-digested pSJH7, forming pSK6.

B22R. pGS120 contains a 3-kb *Bam*HI to *Sal*I fragment from the right end of the *Sal*I fragment of vaccinia virus strain WR (spanning the B22R gene) cloned into pUC13. This 3-kb *Bam*HI to *Sal*I fragment was ligated into *Bam*HI- and *Sal*I-digested pSJH7, generating pNWB12. pSJH7 was partially digested with *Sty*I, a 7-kb fragment was isolated and then digested with *Xma*III, end-filled with Klenow enzyme, and religated. The resultant plas[illegible] t 920 bp (86%) of the B22R ORF es at the 3′ end).

Primer extension analysis

Single-stranded DNA primers (25 nucleotides long, see Figs. 1B and 1C) were 5′ labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. Each oligonucleotide was then hybridized to RNA extracted from cells infected at 15 plaque forming units (PFU) per cell with vaccinia virus strain WR early or late during infection or from mock-infected cells. Early mRNA was prepared from cells 6 hr postinfection (hpi) in the presence of 100 μg/ml cyclohexamide and late RNA 16 hpi in the absence of drugs as previously described (Smith *et al.,* 1989a). Oligonucleotide primers were extended with Superscript RNase H$^-$ reverse transcriptase (Gibco BRL) and the radiolabeled DNA analyzed on a 6% polyacrylamide gel along side an M13 sequencing ladder.

Production of polyclonal antisera

B13R. Two polyclonal rabbit antisera to the B13R protein were produced. The first (anti-B13R-1) was raised against a β-galactosidase-B13R fusion protein containing the C-terminal 74 amino acids of B13R. The *Sal*I I fragment of vaccinia virus strain WR cloned in the *Sal*I site of pUC9 was digested with *Hin*cII and a 202-bp fragment purified and cloned into *Sma*I-digested pEX3 (Stan-

ley and Luzio, 1984) to form plasmid pEX3-202. *E. coli* POPS 2136 cells transformed with pEX3-202 were grown overnight at 30° then diluted 1:100 in fresh medium, grown at 30° for 2 hr, and then expression of the fusion protein induced by growth for 1 hr at 42°. Fusion protein, resolved by SDS–PAGE on a 10% gel, was purified by electroelution from the gel. New Zealand white rabbits were immunized once with 200 μg of fusion protein in Freund's complete adjuvant and subsequently at three-weekly intervals with 100 μg of fusion protein in incomplete Freund's adjuvant. The second serum (anti-B13R-2) was raised against a glutathione *S*-transferase–B13R fusion protein (Smith and Johnson, 1988). A 930-bp *Bam*HI–*Hpa*I fragment, containing the B13R ORF without the 5′ end, was purified from plasmid pYC15 and ligated into plasmid pGEX-2T (Smith and Johnson, 1988), which had been digested with *Eco*R1, end-filled with Klenow enzyme, and then digested with *Bam*HI. The resultant plasmid was called pSK1. The 5′ end of the B13R ORF was reconstructed using polymerase chain reaction (PCR). The PCR product was formed by using oligonucleotides (1) 5′-CCCAGATCTATGGATATCTTCAGG-3′ and (2) 5′-GAGCGCTAACCTTATCC-3′ and pYC15 DNA as template. Oligonucleotide (1) represents the beginning of the B13R ORF and contains a *Bgl*II site (underlined) upstream of the ATG codon. Oligonucleotide (2) is complementary to an internal region of B13R and crosses the *Bam*HI site. The PCR product was digested with *Bgl*II and *Bam*HI and ligated into *Bam*HI-digested pSK1, forming pSK2. The sequence of the region of pSK2 derived by PCR was verified by DNA sequencing. The glutathione *S*-transferase–B13R fusion protein was expressed in *E. coli* strain TG1 transformed with pSK2 by addition of 0.1 m*M* IPTG for 4 hr at 37°. The fusion protein was extracted from the bacterial cell pellet by sonication in phosphate-buffered saline (PBS) followed by centrifugation (6370 *g*, 10 min, 4°) and subsequently purified by application of the soluble fraction to a 2-ml glutathione–Sepharose 4B column (Pharmacia) and elution in 5 m*M* reduced glutathione, 50 m*M* Tris–HCl, pH 8. New Zealand white rabbits were immunized subcutaneously with 300 μg of fusion protein in complete Freund's adjuvant followed by similar injections in incomplete Freund's adjuvant at approximately four-weekly intervals.

B22R. A 1.1-kb *Sty*I to *Ssp*I fragment, which includes a single nucleotide 5′ of the B22R initiation codon and 47 nucleotides 3′ of the termination codon, was purified from pGS120 and ligated into the *Nde*I site of expression vector pGMT7 (obtained from M. Chee, MRC, Laboratory of Molecular Biology, Cambridge, UK) (Hughes *et al.*, 1991). The resultant plasmid, pKL21, was used to transform *E. coli* strain BL21 (DE3)/pLysS and the expression of the B22R protein induced by addition of 1 m*M* IPTG. The B22R protein was purified in inclusion bodies (Hughes *et al.*, 1991) and then resolved on and excised from a 10% polyacrylamide gel. After homogenizing, 100 μg was administered intramuscularly into rabbits in Freund's complete adjuvant followed by subsequent boosts in incomplete Freund's adjuvant given at six-weekly intervals.

Immunoblotting

Monolayers of BSC-1 cells were infected at 10 PFU/cell. At 16 hpi cell monolayers were scraped, collected by centrifugation, and resuspended in 200 μl protein loading buffer (Laemmli, 1970). Proteins were resolved by SDS–PAGE (10% gel), transferred to a nitrocellulose membrane (Towbin *et al.*, 1979), and incubated with the polyclonal rabbit anti-B13R-2 serum. For colorimetric detection the anti-B13R serum was used at 1:200 dilution. Bound immunoglobulin (Ig) was detected by incubation with anti-rabbit IgG conjugated to alkaline phosphatase (Sigma) followed by reaction with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium. For detection by enhanced chemiluminescence (ECL) (Amersham), the anti-B13R serum was used at 1:3000 dilution and bound Ig was detected by incubation with anti-rabbit IgG horse rabbit peroxidase followed by detection using the ECL reagents as described by the manufacturer.

Immunoprecipitation

BSC-1 cells were infected with virus at either 30 or 50 PFU/cell. After 1 hr the virus inoculum was removed and the cells were incubated in MEM with 2.5% FBS. At the appropriate time thereafter the medium was removed and the cells were washed in MEM lacking methionine and cysteine and then incubated in the same. After 30 min the medium was supplemented with 100 μCi of Trans [^{35}S] label (ICN Biomedicals: a mixture of approximately 80% [^{35}S]methionine and 20% [^{35}S]cysteine) and incubation continued for the appropriate time. The cells were then washed in PBS and lysed by the addition of 0.5 ml IP buffer (10 m*M* Tris–HCl, pH 7.5, 150 m*M* NaCl, 1% sodium deoxycholate, 1% NP-40, 0.1% SDS, 1 mg/ml BSA, 0.02% NaN_3, and 1 m*M* PMSF). Cell lysates were cleared by centrifugation for 15 min in a microfuge and 200 μl of supernatant was incubated with 10 μl of 25% (v/v) protein A–Sepharose at 4° for 2 hr. The protein A–Sepharose was removed by low speed centrifugation and the supernatant incubated with 10 μl polyclonal rabbit anti-serpin serum at 4° overnight. Immune complexes were collected by incubation with 50 μl protein A–Sepharose for 2 hr and then washed three times with IP buffer. Bound proteins were resolved by SDS–PAGE (10% gel) and detected by fluorography.

Construction of virus deletion mutants and revertants

Virus deletion mutants lacking the B13R or B22R ORF were constructed by transient dominant selection (Falk-

ner and Moss, 1990) using the Ecogpt gene as the transient selectable marker (Boyle and Coupar, 1988; Falkner and Moss, 1988). CV-1 cells were infected with vaccinia WR strain at 0.01 PFU/cell and transfected with calcium phosphate-precipitated pSK5 DNA (for the construction of the B13R deletion mutant) or pΔB22R DNA (for the B22R deletion mutant). Progeny virus harvested 2 days later was plated onto BSC-1 cells pretreated for 3 to 12 hr with 25 μg/ml mycophenolic acid (MPA), 250 μg/ml xanthine, and 15 μg/ml hypoxanthine and then maintained in the presence of these drugs (Boyle and Coupar, 1988; Falkner and Moss, 1988). Plaque isolates were purified two or three times under selection and then three times without selection to resolve the MPA-resistant intermediate plaques, producing the deletion mutants and sibling WT viruses. Virus isolates were screened by PCR and Southern blot analysis and were then amplified and purified. The B13R deletion mutant was named ΔB13R and the sibling WT virus named B13RWT. Similarly, for B22R, the viruses were named ΔB22R and B22RWT. Revertant viruses were constructed using transient dominant selection as described above. For B13R, CV-1 cells were infected with ΔB13R at 0.01 PFU/cell and transfected with calcium phosphate-precipitated pSK6. The MPA-resistant intermediate plaque was resolved to produce the original deletion mutant and a revertant virus named B13Rrev in which the B13R locus had been restored to that of WT virus. Similarly for B22R, CV-1 cells were infected with ΔB22R and transfected with pNWB12. After the resolution of the MPA-resistant intermediate plaque, the virus in which the B22R locus had been restored was named B22Rrev. The genomic structures of all viruses were checked by PCR and Southern blot analysis.

Virulence assay

Groups of five, 5- to 6-week-old, female, Balb/c mice weighing between 15 and 20 g were anesthetized and infected intranasally with virus doses ranging from 10^4 to 10^6 PFU in 25 μl of PBS, pH 7.2. Aliquots of the viruses used for infection were titrated on BSC-1 cells to confirm the dose administered. Animals were individually weighed daily. Animals which had lost greater than 30% of their original body weight were sacrificed by cervical dislocation.

RESULTS

Transcriptional analyses

From inspection of the nucleotide sequence it was predicted that ORFs B13R and B22R would be transcribed at early times during infection (Smith *et al.*, 1989b). They are not preceded by the conserved TAAATG motif found at the transcription initiation site of most late genes (Rosel *et al.*, 1986), and there are early transcription termination sequences, T_5NT (Yuen and Moss, 1987), immediately after B13R and 25 nucleotides before the 3′ end of B22R. Northern blot analyses of vaccinia mRNA had confirmed that these ORFs are transcribed early (Smith *et al.*, 1989b). To more accurately map the 5′ ends of the B13R and B22R mRNAs, primer extension analyses were performed.

For B13R, extension of the primer (detailed in Fig. 1B) produced major extension products of 96 and 97 nucleotides with RNA extracted from cells early during infection but no products were detected with RNA extracted from cells late during infection or from mock-infected cells (Fig. 1A). Thus it was determined that the B13R early mRNA initiates 71–72 nucleotides upstream of the initiation codon. S1 nuclease protection experiments mapped the 5′ end of B13R early transcripts to 72–73 nucleotides upstream of the initiation codon (data not shown). For B22R, extension of the primer (detailed in Fig. 1C) produced a major extension product of 72 nucleotides with RNA extracted from infected cells early during infection but no products were detected with RNA extracted from cells late during infection or from mock-infected cells. The B22R early mRNA therefore initiates 31 nucleotides upstream of the initiation codon (Fig. 1C). Each early transcript presumably terminates just after a T_5NT motif which is present either immediately downstream of the ORF (B13R) or near the 3′ end of the ORF (B22R) to produce transcripts of sizes consistent with those determined by Northern analysis (Smith *et al.*, 1989b).

Identification of the B13R and B22R polypeptides

To identify the B13R and B22R proteins in vaccinia virus-infected cells a rabbit antiserum was raised against each of the ORFs expressed in *E. coli* (Materials and Methods). Immunoblot analysis (Fig. 2A) using anti-B13R-2 serum detected a M_r 38.5K protein in cells infected with WR (lane 2) but not in mock-infected cells (lane 1) nor cells infected with the deletion mutant ΔB13R described under Materials and Methods and below (lane 4). The anti-B22R serum immunoprecipitated a M_r 40K protein from cells infected with WR (Fig. 2B, lane 2) but not from mock-infected cells (lane 1) nor cells infected with the deletion mutant ΔB22R (lane 4). In each case the serpin was detected in cells infected with the revertant virus (Figs. 2A and 2B, lane 5) and in cells infected with a plaque-purified WT virus isolated by transient dominant selection during the construction of the deletion mutants (Figs. 2A and 2B, lane 3). When the infections were repeated in the presence of tunicamycin, an inhibitor of N-linked glycosylation, or monensin, an inhibitor of O-linked glycosylation and vesicular transport, the size of the proteins detected were unaltered indicating that these proteins are probably nonglycosylated (data not shown).

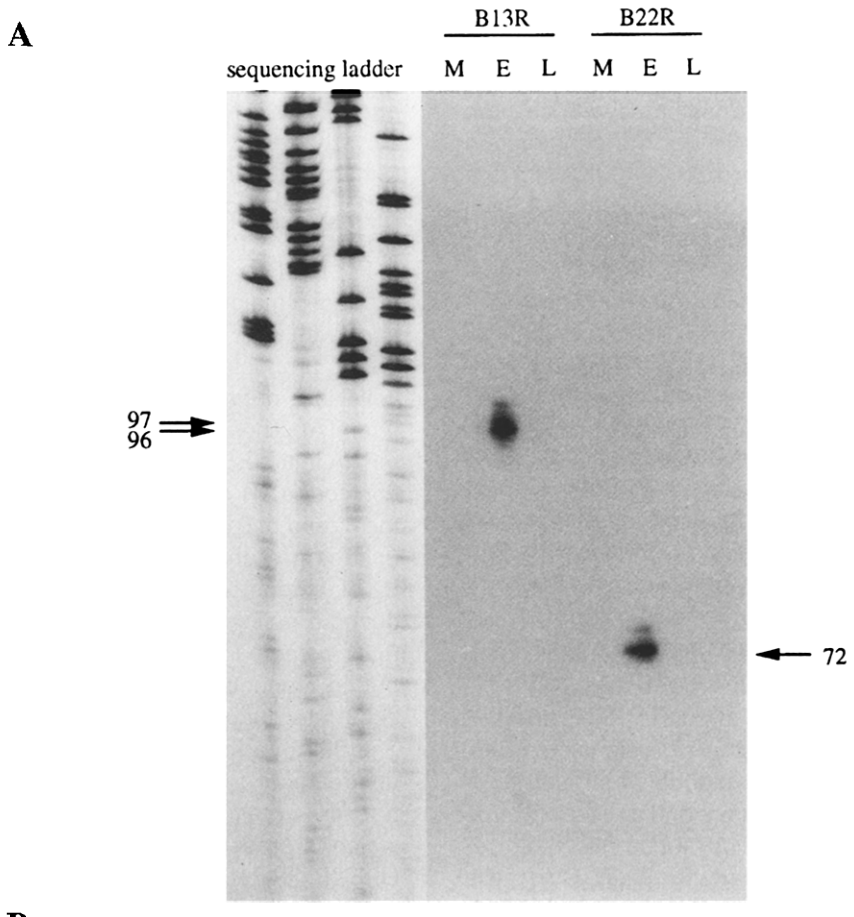

FIG. 1. Primer extension analyses to map the 5′ ends of the B13R and B22R mRNAs. (A) RNA extracted from mock-infected cells (M), or from cells early (E) or late (L) after infection with vaccinia virus, was hybridized to the ^{32}P-labeled primers detailed in (B) for B13R and (C) for B22R. After reverse transcription, the extension products were run on a 6% polyacrylamide gel alongside a sequencing ladder. An autoradiogram is shown. (B) Position of the deduced 5′ end of the B13R early mRNA (●). The oligonucleotide primer used for extension is underlined. (C) Position of the deduced 5′ end of the B22R early mRNA (●). The oligonucleotide used for reverse transcription is underlined.

The stability and location of the serpins was analyzed by pulse–chase experiments. WR-infected cells were labeled with [^{35}S]methionine and [^{35}S]cysteine from 2.5 to 3.5 hpi and then chased for various times up to 24 hpi. Cell extracts prepared at these times, or immediately after pulse-labeling, were immunoprecipitated with anti-B13R-1 (Fig. 2C) or anti-B22R sera (Fig. 2D). These data show that each protein is reasonably stable within infected cells and remains detectable at 24 hpi. Analysis of the proteins present in the tissue culture supernatant indicated that neither serpin was secreted from the cell (data not shown).

Presence of serpins in other orthopoxviruses

The presence of the B13R and B22R gene products in other orthopoxviruses was investigated by immunoblot analysis for B13R (Fig. 3B) and by immunoprecipitation

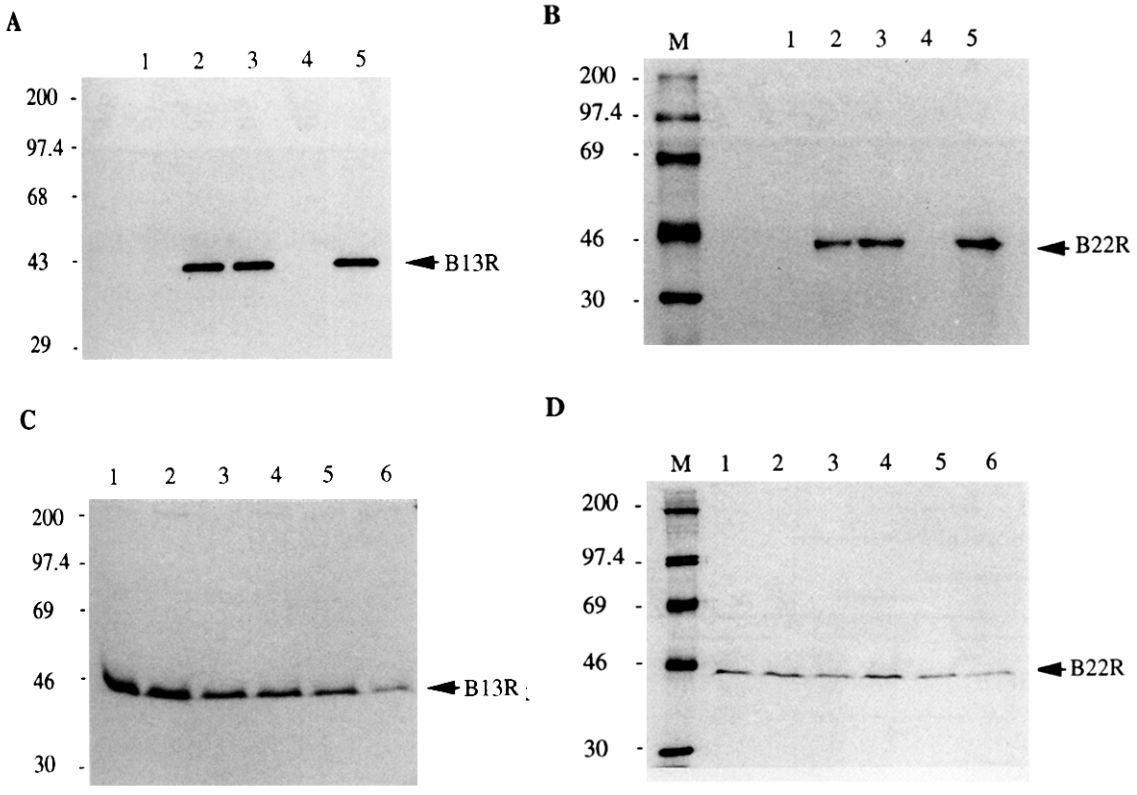

FIG. 2. (A) Immunoblot analysis of the B13R protein. Monolayers of BSC-1 cells were mock-infected (lane 1) or infected with WR (lane 2), B13RWT (lane 3), ΔB13R (lane 4), or B13Rrev (lane 5) at 10 PFU/cell. Extracts from cells harvested 16 hpi were resolved on a 10% polyacrylamide gel, transferred to nitrocellulose, and reacted with anti-B13R-2-specific serum, and bound Ig was detected using ECL reagents (see Materials and Methods). Molecular weights are indicated in kDa. (B) Immunoprecipitation of the B22R protein. BSC-1 monolayers were mock-infected (lane 1) or infected with WR (lane 2), B22RWT (lane 3), ΔB22R (lane 4), or B22Rrev (lane 5) at 30 PFU/cell and labeled with a mixture of [^{35}S]methionine and [^{35}S]cysteine between 2.5 and 4.5 hpi. Cell lysates were immunoprecipitated with B22R-specific serum, the immunoprecipitates resolved on a 10% polyacrylamide gel and detected by fluorography. Molecular weight markers are shown in kDa (lane M). (C and D) Pulse–chase analysis. BSC-1 monolayers were infected with WR at 30 PFU/cell and labeled between 2.5 and 3.5 hpi. Lysates were prepared immediately after pulse (lane 1) or after chase in GMEM containing 2.5% FBS for 1 (lane 2), 2.5 (lane 3), 4.5 (lane 4), 8.5 (lane 5), and 20.5 hr (lane 6) and immunoprecipitated with anti-B13R-1 (C) or anti-B22R (D) serum and then resolved by SDS–PAGE on a 10% gel and detected by fluorography. Molecular weight markers are shown in kDa.

for B22R (Fig. 3A). For B13R, a protein of approximately M_r 38.5K was detected in cells infected with vaccinia virus strains WR and IHD-J, while slightly smaller proteins were found in cells infected with rabbitpox virus, cowpox virus, and vaccinia virus strain Wyeth (Fig. 3B). A protein of the same size as the IHD-J B13R protein was also found in cells infected with IHD-W (data not shown). The sequence data for the cowpox virus 38 K gene predicts a smaller protein than that in vaccinia virus WR (Pickup *et al.*, 1986; Smith *et al.*, 1989b). The failure to detect the B13R protein in vaccinia virus Copenhagen is consistent with published sequence data in which there are deletions corresponding to the 5′ end and an internal region of the WR ORF that lead to an N-terminal truncation and downstream frameshift mutation, respectively (Goebel *et al.*, 1990; Smith *et al.*, 1991). Given that the B13R protein was only detected in cells infected by some of the viruses, it was necessary to show that similar levels of virus protein were present in all samples. This was done by immunoblotting with B5R-specific antiserum (Engelstad *et al.*, 1992), since the extracellular enveloped virus (EEV) gp42 protein encoded by B5R is expressed by all the viruses used above (Engelstad and Smith, 1993) (data not shown). In contrast to the situation with B13R, a B22R protein was expressed by all the orthopoxviruses examined (Fig. 3A).

Construction and characterization of virus deletion mutants and revertants

Virus deletion mutants which lack either B13R or B22R (ΔB13R and ΔB22R) and revertant viruses in which the gene was reinserted (B13Rrev and B22Rrev) were constructed as described under Materials and Methods. During the construction of the deletion mutants by transient dominant selection, plaque-purified WT viruses (B13RWT and B22RWT) were also isolated that are genetically closer to the deletion mutants than the uncloned

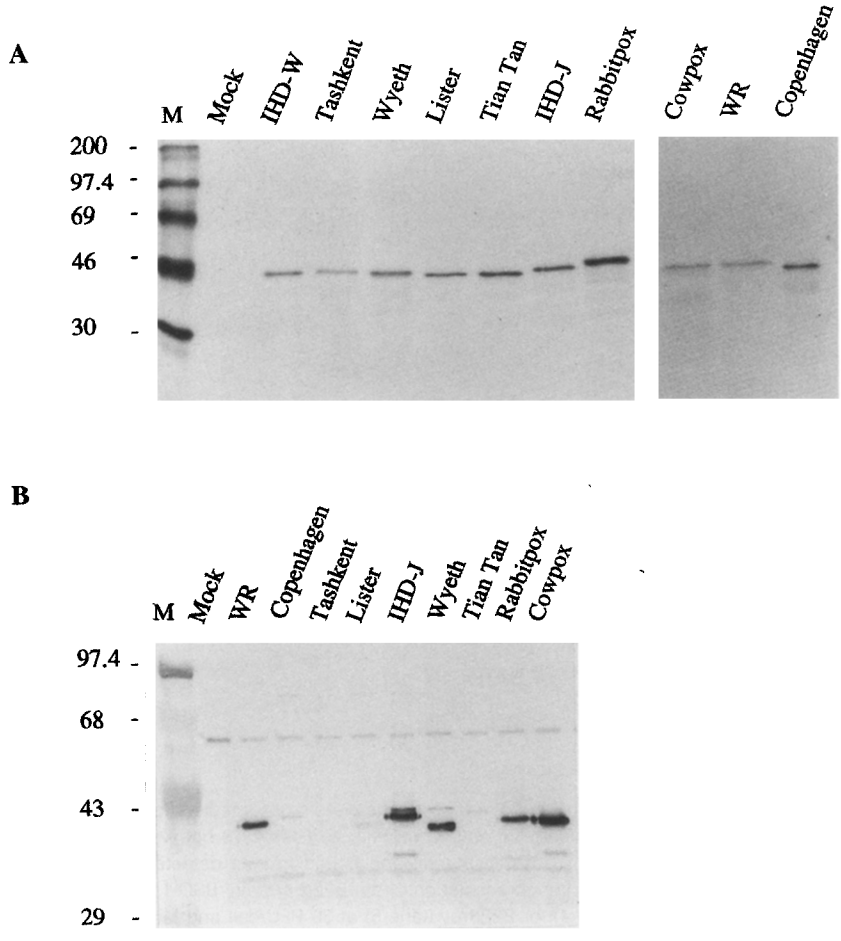

FIG. 3. Distribution of the B13R and B22R proteins in orthopoxviruses. (A) B22R. BSC-1 monolayers were infected with the indicated orthopoxvirus at 30 PFU/cell and radiolabeled with [^{35}S]methionine and [^{35}S]cysteine, and extracts immunoprecipitated with anti-B22R serum as described in the legend to Fig. 2B. A fluorogram is shown and molecular weight markers are indicated in kDa (lane M). (B) B13R. BSC-1 monolayers were infected with the various orthopoxviruses at 10 PFU/cell for 16 hr and then treated as described in the legend to Fig. 2A except that bound Ig was detected using colorimetric assay rather than ECL (see Materials and Methods). Molecular weight markers are indicated in kDa (lane M).

WR stock. The genomic structures of these viruses are illustrated in Figs. 4 and 5 together with PCR and Southern blotting data confirming their structures.

Previous experience in our laboratory has shown that when the 7.5K promoter driving the Ecogpt gene is inserted into genes within the *Hin*dIII B fragment in the same orientation as the native 7.5K gene within the proximal inverted terminal repeat (ITR), intramolecular recombination events between these copies of the 7.5K promoter may occur leading to large deletions (S.K., N.W.B., and A. Alcamí, unpublished data). We have therefore used PCR with oligonucleotides that amplify the genes to the left and right of the B13R and B22R genes as well as the B13R and B22R loci themselves, to ensure that the genomes of the recombinant viruses are as expected. Figure 4B shows that oligonucleotides from genes B12R or B14R amplify the same size fragments from each virus (270 or 449 bp, respectively), whereas oligonucleotides that span both the B13R and B14R genes produce a WT fragment of 1561 bp from WR, B13RWT, and B13Rrev viruses, but a smaller fragment of 635 bp from ΔB13R. Similarly, for the B22R viruses (Fig. 5B), PCR products for the B21R or B23R genes were the same for all viruses (408 and 578 bp, respectively), whereas oligonucleotides amplifying the B22R gene produced a fragment of 1148 bp for WR, B22RWT, and B22Rrev, but a smaller fragment of 228 bp for ΔB22R (Fig. 5B).

In addition, Southern blotting *Sal*I- or *Bam*HI-digested virus DNA with probes that represent the region of B13R or B22R deleted in ΔB13R or ΔB22R viruses, respectively (marked Δ in Figs. 4C and 5C), detected bands of the expected sizes with all viruses except the corresponding deletion mutant where no hybridization was detected (Figs. 4A and 5A). Similarly, blotting *Ssp*I- or *Sal*I-digested virus DNA with probes from the 3′ flanking region of the B13R or B22R genes (marked F in Figs. 4C and 5C) identified fragments of WT size in all viruses except the deletion mutants where the band was reduced

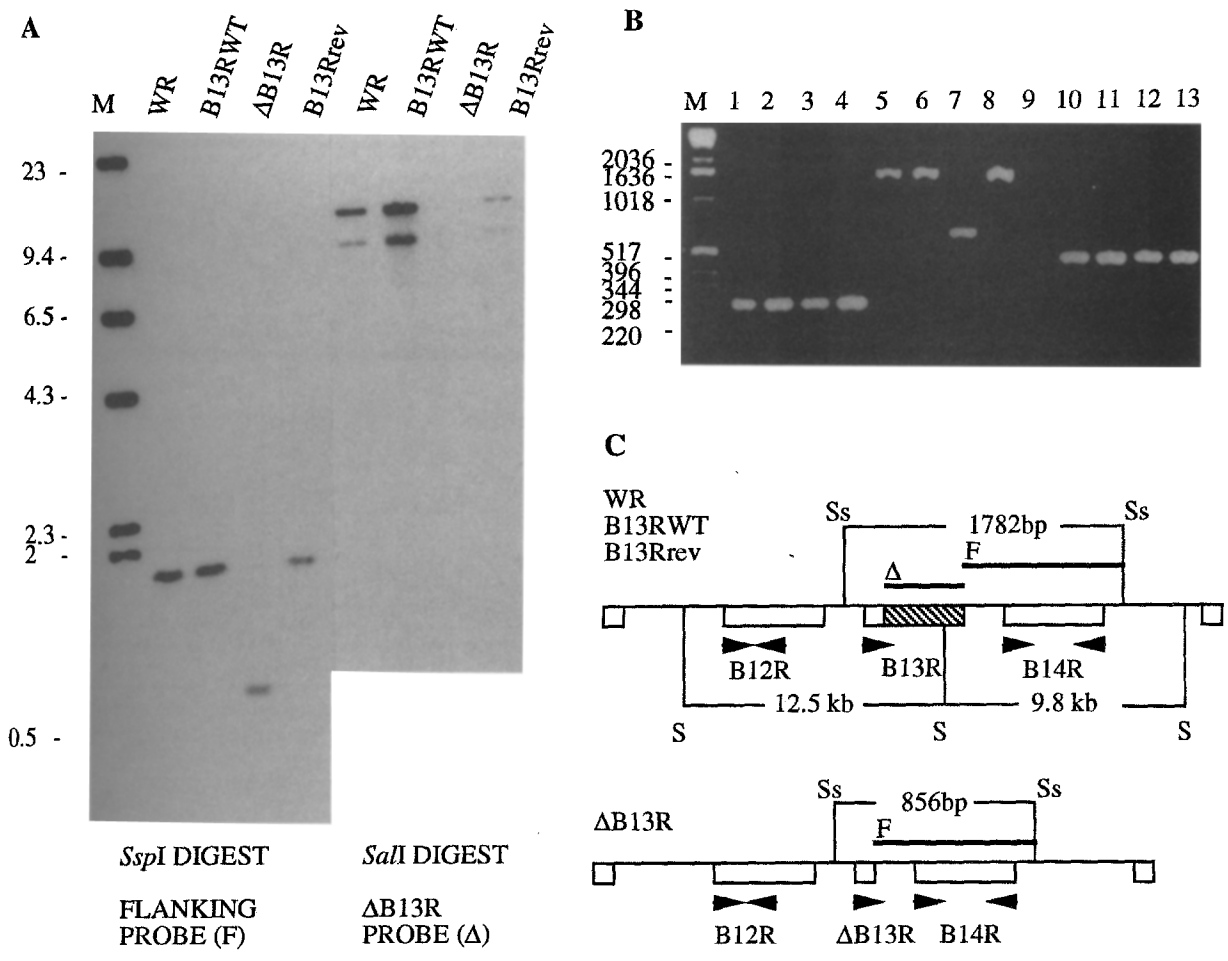

Fig. 4. Genomic structure of recombinant B13R viruses. (A) Southern blot. DNA isolated from the core of viruses WR, B13RWT, ΔB13R, or B13Rrev was digested with *Sal*I or *Ssp*I and the fragments were resolved on a 0.7% agarose gel before transfer to nitrocellulose. Filters were probed with ECL-labeled probes representing the sequence deleted from the endogenous B13R gene (Δ) or a fragment from the 3′ flanking region (F) (shown in C). Sizes of the ECL-labeled markers are shown in kb. (B) PCR analysis. DNA from WR (lanes 1, 5, and 10), B13RWT (lanes 2, 6, and 11), ΔB13R (lanes 3, 7, and 12), or B13R rev (lanes 4, 8, and 13) was used in PCR and the resultant DNA fragments resolved on an agarose gel. The pairs of oligonucleotide primers used were (a) from the 5′ end of the B12R ORF and 270 bp from the 5′ end (lanes 1–4), (b) from the 5′ end of B13R and from the 3′ end of B14R (lanes 5–8), and (c) from the 5′ and 3′ ends of B14R (lanes 10–13). Lane 9 was a no DNA control for primers used in lanes 5–8. Markers are shown in bp. (C) The relevant *Sal*I (S) and *Ssp*I (Ss) fragments containing the B13R gene (not to scale). The probes used in the Southern blot analyses (Δ and F) as well as the primers used for the PCR analysis (arrows) are shown. The region deleted in ΔB13R is represented by a hatched box.

in size as expected. These data show that the structures of the virus genomes were as predicted and no alterations in the *Hin*dIII B region had arisen other than in B13R or B22R genes.

The isolation of the deletion mutants ΔB13R and ΔB22R viruses confirms that B13R and B22R are not required for virus replication *in vitro* (Zhou *et al.*, 1990) and is consistent with the observed lack of the B13R protein expression in several viruses (above). To more accurately measure the growth properties of WR and the deletion mutants, low multiplicity of infection (m.o.i.) (0.01 PFU/cell) growth curves were performed in BSC-1 cells (Figs. 6A and 6B). The results showed that the growth kinetics and final titers of the deletion mutants were indistinguishable from WR.

Vaccinia virus produces two forms of infectious virus: intracellular mature virus (IMV) and EEV. EEV represents approximately only 1% of total infectious virus with the WR strain and, therefore, alterations in EEV formation would be masked in measurements of total virus. A second virus growth experiment, this time at high m.o.i. (10 PFU/cell) was, therefore, undertaken (Figs. 6C and 6D). IMV and EEV present within infected cells or in the culture supernatant, respectively, at 24 hpi were titrated on BSC-1 cells. The loss of either serpin gene did not affect the levels of IMV and EEV produced.

Virulence of deletion mutants

The involvement of some orthopoxvirus serpins in virus pathogenesis led us to investigate whether vaccinia virus

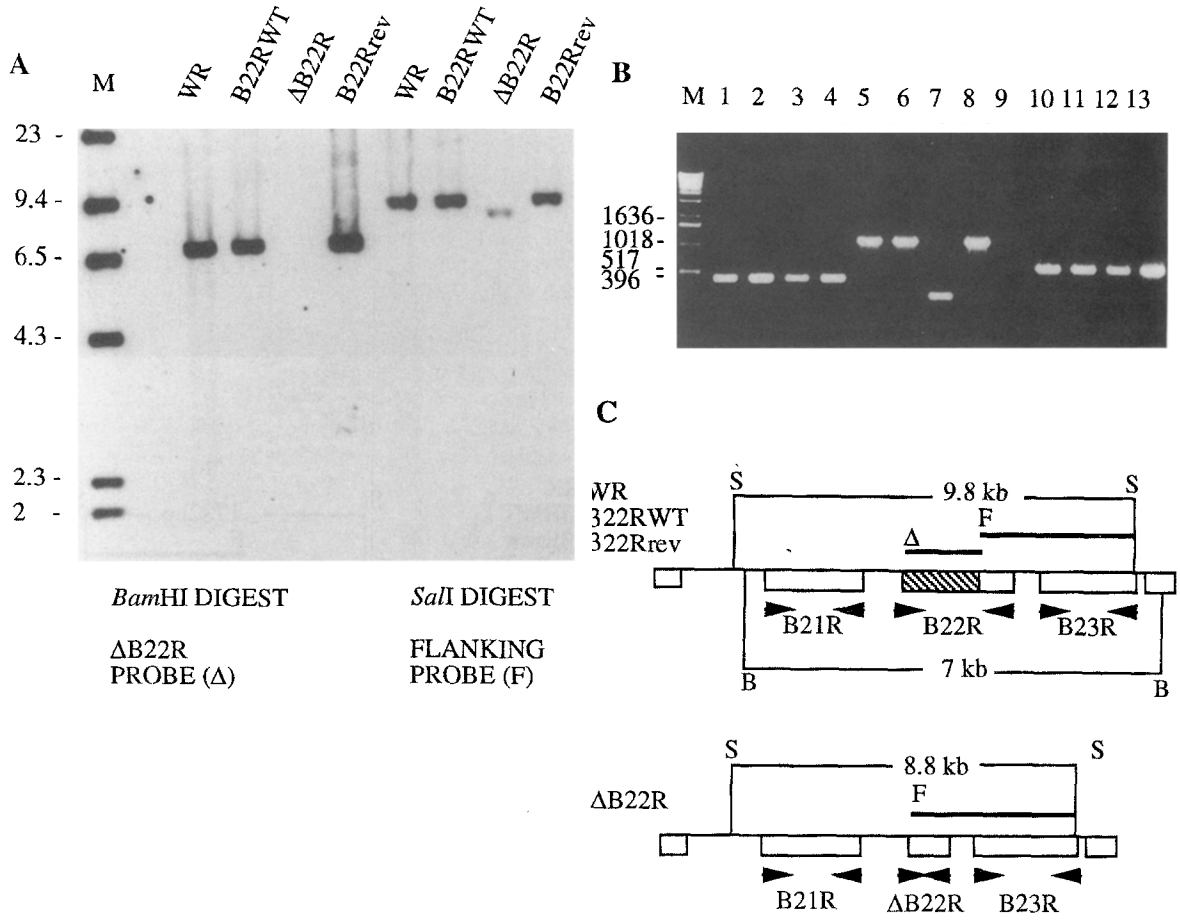

FIG. 5. Genomic structure of recombinant B22R viruses. (Methods as described in the legend to Fig. 4.) (A) Southern blot analyses. DNA from WR, B22RWT, ΔB22R, or B22Rrev was digested with *Bam*HI or *Sal*I and probed with the sequence representing the region deleted from the endogenous B22R gene (Δ) or a fragment from the 3′ flanking region of B22R (F). Sizes of the ECL-labeled markers are shown in kb. (B) PCR analysis. DNA from WR (lanes 1, 5, and 10), B22RWT (lanes 2, 6, and 11), ΔB22R (lanes 3, 7, and 12), or B22Rrev (lanes 4, 8, and 13) was used in PCR. Primers used in the analysis were (a) from the 5′ and 3′ of B21R (lanes 1–4), (b) from just upstream of B22R and the 3′ end of B22R (lanes 5–8), and (c) from the 5′ and 3′ ends of B23R (lanes 10–13). Lane 9 was a no DNA control with the primers used in lanes 5–8. Sizes of the markers are shown in bp. (C) The relevant *Sal*I (S) and *Bam*HI (B) fragments containing the B22R gene (not to scale). The probes used in the Southern blot analysis (Δ and F) as well as the primers used for the PCR analysis (arrows) are shown. The region deleted in ΔB22R is represented by a hatched box.

WR serpins B13R and B22R contribute to virus virulence in a murine intranasal model. For these experiments we used the plaque-purified WT viruses (B13RWT and B22RWT) that were isolated during the construction of the deletion mutants, the deletion mutants (ΔB13R and ΔB22R), and the revertant viruses (B13Rrev and B22Rrev). With all viruses, infections at 10^5 PFU or greater caused a rapid and severe weight loss and all animals were sacrificed by Day 7 postinfection by which time they had lost at least 30% of their body weight (Figs. 7B, 7C, 7E, and 7F). At 10^4 PFU there were no differences in the amount of weight lost or the rate of recovery between mice infected with ΔB13R, B13Rrev, or B13RWT (Fig. 7A). Similarly, there was no difference between ΔB22R and B22RWT and the slightly greater weight loss of B22Rrev was due to a 2.5-fold increased virus input compared to the other viruses (Fig. 7D). Overall, these results show that deleting B13R or B22R does not attenuate the virus in this model system.

DISCUSSION

In this paper a characterization of vaccinia virus (WR strain) genes B13R and B22R is presented. Transcriptional analyses showed that early mRNAs initiate 71 to 72 and 31 nucleotides upstream of the B13R and B22R ORFs, respectively. The length of these 5′ untranslated regions is longer than is the case for many vaccinia virus mRNAs but in each case there were no other AUG codons between the 5′ end of the RNA and the AUG of the serpin ORF. The sequences upstream of the early RNA start sites showed some similarity with the consen-

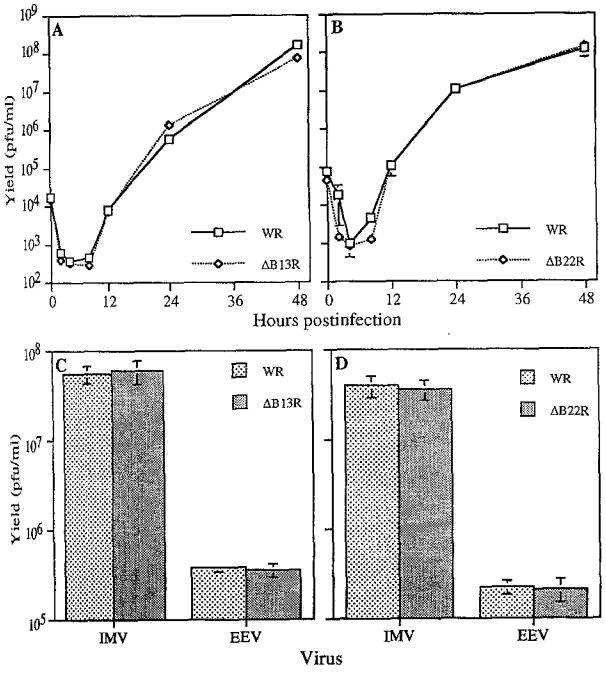

FIG. 6. Growth curves of WR and serpin deletion mutants. (A and B) Duplicate monolayers of BSC-1 cells were infected at 0.01 PFU/cell with WR or ΔB13R (A) or with WR or ΔB22R (B). After 1 hr unbound virus was removed by washing with PBS and the cells were incubated in DMEM containing 2.5% FBS. At indicated times postinfection the cells were scraped into the growth medium, freeze–thawed three times, sonicated, and then titrated on BSC-1 cells. The data shown are the mean values from duplicate samples. (C and D) IMV and EEV formation by WR and serpin deletion mutants. Triplicate monolayers of BSC-1 cells were infected at 10 PFU/cell with WR or ΔB13R (C) or with WR or ΔB22R (D). After 1 hr unbound virus was removed by washing with PBS and the cells were incubated in DMEM containing 2.5% FBS. After 24 hr the culture medium was centrifuged (500 *g*, 5 min, 4°) to pellet any detached cells and the supernatant retained as the EEV sample. For IMV the cells were washed in PBS, scraped into PBS, freeze–thawed three times, and sonicated. EEV and IMV samples were titrated on BSC-1 cells. The data shown are the mean values from triplicate samples.

sus for early vaccinia virus promoters (Davison and Moss, 1989).

The proteins encoded by the B13R and B22R ORFs of vaccinia virus strain WR were identified as M_r 38.5 and 40K intracellular polypeptides by the use of specific antisera raised against bacterially expressed proteins. Each protein was stable within the infected cell, was not secreted into the medium, and was not altered by addition of the glycosylation inhibitors tunicamycin and monensin. The B13R gene product was only detected in vaccinia virus strains WR, IHD-J, IHD-W, and Wyeth and in rabbitpox and cowpox and had a slightly reduced size in the latter three cases. In contrast, the B22R gene product was detected in all strains tested including Copenhagen, Tian Tan, Lister, and Tashkent (strains not expressing B13R). This distribution may reflect the relative importance of the genes *in vivo*.

Viable deletion mutants lacking the majority of the B13R or B22R ORFs were constructed. Earlier attempts to isolate such recombinant viruses by insertion of the 7.5K promoter–Ecogpt cassette into the serpin loci in the same orientation as the 7.5K gene in the proximal ITR had resulted in large deletions presumably by intramolecular recombination between the duplicated promoter sequences. Such use of promoters that are repeated in genes which are separated by only nonessential genes may reflect a general difficulty in the construction of recombinant poxviruses. Analysis of the authentic deletion mutants showed that they had unaltered growth kinetics *in vitro* and were not attenuated *in vivo* in a murine intranasal model. For B22R the latter result is consistent with that reported for deletion mutants in the corresponding gene of cowpox and rabbitpox virus (termed SPI-1) (Thompson *et al.*, 1993). However, for B13R our results differ from those of Thompson *et al.* (1993), who observed striking attenuation when B13R was deleted in cowpox and rabbitpox. The reason for this discrepancy is unclear, but it may reflect genuine differences in the viruses (unlike cowpox and rabbitpox the WR virus is highly neurotropic). Alternatively, mutations might have been acquired elsewhere in the virus genomes during the isolation of the cowpox or rabbitpox deletion mutants which might explain the difference. The production of cowpox or rabbitpox revertant viruses from these deletion mutants would address this possibility.

The function of the B22R serpin is unknown but its expression in all orthopoxviruses tested and the conservation of the gene sequence in two strains of variola virus (Massung *et al.*, 1993; Shchelkunov *et al.*, 1993) and ectromelia virus (Senkevich *et al.*, 1993) suggests it has an important role in orthopoxvirus replication in animals. It has been suggested that a role for the vaccinia serpins may be to inhibit the cellular proteinases responsible for the degradation of antigens into peptides for presentation to cytotoxic T lymphocytes (CTLs) in association with class I major histocompatibility (MHC) antigens (Smith *et al.*, 1989b). This would reduce the presentation of virus-specific peptides to CTLs and decrease the recognition and clearance of infected cells. However, recent data suggest that the B13R and B22R serpins of vaccinia WR do not block the presentation of several influenza virus peptides to class I MHC-restricted CTL during infection with vaccinia (unpublished data). Nonetheless the deletion of B13R and B22R from vaccinia strain WR potentiated the antibody and CTL responses to the human papillomavirus type 16 L1 protein expressed by recombinant vaccinia viruses (Zhou *et al.*, 1990, 1991).

Several possible functions for the cowpox equivalent of the vaccinia virus B13R protein have been proposed (see Introduction). Since the B13R protein shares 92% amino acid identity with the cowpox protein it would be

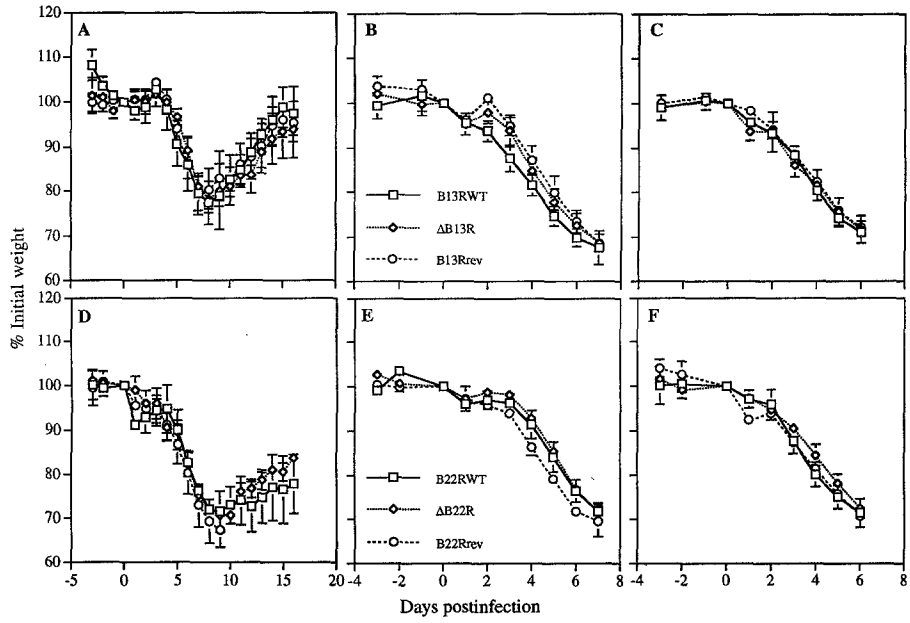

FIG. 7. Weight loss of mice infected with recombinant vaccinia viruses. Groups of five, 5- to 6-week-old, female, Balb/c mice were infected intranasally either with B13RWT, ΔB13R, or B13Rrev (A–C) (symbols shown in B) or with B22RWT, ΔB22R, or B22Rrev (D–F) (symbols shown in E) and weighed individually before infection and daily thereafter. On each day the weight of each mouse was expressed as the proportion of its weight on Day 0 and the mean of each group of five calculated. Mice were infected with 10^4 (A and D), 10^5 (B and E), or 10^6 (C and F) PFU of virus. Animals infected with doses of 10^6 or 10^5 were sacrificed on Days 6 and 7, respectively.

expected to have similar functions. However, despite the presence of a similar B13R gene (38 K, SPI-1, or crmA) in both vaccinia and cowpox, during cowpox infection of chick CAMs the 38 K protein is a potent inhibitor of the inflammatory response (Pickup *et al.*, 1986; Palumbo *et al.*, 1994), while during vaccinia infection there is little or no inhibition of the inflammatory response (Palumbo *et al.*, 1994). Additionally, deletion of the 38 K serpin from cowpox causes attenuation (Thompson *et al.*, 1993), whereas this study shows that deletion of the vaccinia serpin B13R does not. Clearly, many different molecules and pathways are involved in the host response to virus infection and orthopoxvirus pathogenesis is multifactorial. It may be that the pathways inhibited by the B13R protein are less important during vaccinia virus infection than during cowpox infection, or vaccinia may encode other proteins which inhibit these pathways via an alternative route.

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